

Demonstration of three proteins induced by vitamin K absence (PIVKAs)

Citation for published version (APA):

Hemker, H. C., Lindhout, M. J., & Reekers, P. P. M. (1973). Demonstration of three proteins induced by vitamin K absence (PIVKAs). In E. Deutsch, K. M. Brinkhous, K. Lechner, & S. Hinnom (Eds.), *Thrombosis: Pathogenesis and Clinical Trials: Transactions of the Plenary Sessions, IV International Congress on Thrombosis and Haemostasis, and Proceedings, International Committee on Thrombosis and Haemostasis, Vienna, Austria, June 18 to 23, 1973* (1 ed., pp. 47-52). F. K. Schattauer Verlag.

Document status and date:

Published: 01/01/1973

Document Version:

Other version

Please check the document version of this publication:

- A submitted manuscript is the version of the article upon submission and before peer-review. There can be important differences between the submitted version and the official published version of record. People interested in the research are advised to contact the author for the final version of the publication, or visit the DOI to the publisher's website.
- The final author version and the galley proof are versions of the publication after peer review.
- The final published version features the final layout of the paper including the volume, issue and page numbers.

[Link to publication](#)

General rights

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal.

If the publication is distributed under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license above, please follow below link for the End User Agreement:

www.umlib.nl/taverne-license

Take down policy

If you believe that this document breaches copyright please contact us at:

repository@maastrichtuniversity.nl

providing details and we will investigate your claim.

Reprint from

Thrombosis: Pathogenesis and Clinical Trials

Transactions of the Plenary Sessions, IV International Congress on Thrombosis
and Haemostasis, and Proceedings, International Committee on Thrombosis and
Haemostasis

Vienna, Austria, June 18 to 23, 1973

E. DEUTSCH

Co-Editor
President,
International Society

K. M. BRINKHOUS

Co-Editor
Secretary-General,
International Committee

K. LECHNER

Co-Editor
Vice-President,
International Society

S. HINNOM

Associate Editor

106 Figures, 56 Tables



F. K. SCHATTAUER VERLAG · STUTTGART-NEW YORK

4. Demonstration of Three Proteins Induced by Vitamin K Absence (PIVKAs)

H. C. HEMKER, M. J. LINDHOUT and P. P. M. REEKERS

Summary

Three different types of proteins induced by vitamin K absence (PIVKAs), corresponding to the normal factors II, IX and X, respectively, have been demonstrated by the use of specific antibodies against these three factors. The PIVKAs could be isolated in small quantities by immunoadsorption techniques.

Key words: PIVKA; precursors, factors II, IX, X; vitamin K deficiency; coumarins.

★

There are certain analogies between the discovery of the planet Neptune and that of the *Proteins Induced by Vitamin K Absence* (PIVKA, for short). As you know, the astronomers Adams and Leverrier inferred the existence of Neptune from otherwise unexplainable deviations in the behavior of the planets known at that time, and then used mathematical procedures to find out where to look for it. In a comparable way we observed deviations from the normal kinetic pattern in the results of tests of the prothrombin-time type carried out on the plasmas of orally anticoagulated patients. The kind of discrepancies we found are illustrated in Table 1. The percentage of coagulation factors read from an overall

Table 1. Determination of individual coagulation factors compared with Thrombotest.*

	II	VII	IX	IX	MEAN	TT
Parenchymatous liver disease (n = 29)	34.5	37.4	34.4	39.7	36.5	37.3
Deep anticoagulation (n = 50)	12.5	13.8	12.0	12.0	12.6	6.5
Moderate anticoagulation (n = 100)	15.0	15.4	17.2	15.0	15.6	8.0
Superficial anticoagulation (n = 50)	19.7	20.4	22.6	20.6	20.8	10.0
Vitamin K deficiency (n = 14)	36.9	38.7	30.8	35.5	35.5	19.3

* The values represent the observed level as a percentage of normal pooled plasma. Reference curves were obtained by dilution of normal pooled plasma with Al(OH)₃-adsorbed plasma.

Laboratory of Cardiovascular and Blood Coagulation Biochemistry, Department of Internal Medicine, University Medical Centre, Leiden, The Netherlands.

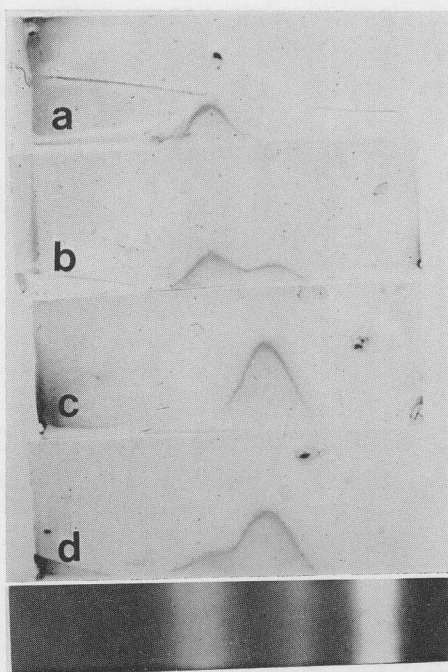


Fig. 4. Two-dimensional crossed electrophoresis of bovine plasmas in the presence of Lactate (first dimension, horizontal) and anti-factor IX antiserum (second dimension, vertical). a: normal plasma. b: plasma after 24 h of anticoagulation. c: plasma after 100 h of anticoagulation. d: equal parts of a and c.

vitamin K causes a block in the synthesis of prothrombin in a postribosomal stage. We argued that a polypeptide precursor that was unable to pass this final stage piled up intracellularly and subsequently reached the circulation. If this hypothesis were true, one would expect four PIVKAs to exist, because the four vitamin K dependent coagulation factors have a different primary polypeptide chain structure. A postribosomal step in the synthesis will not involve the primary structure. This makes it probable that for each of the four vitamin K dependent factors there is a corresponding PIVKA, which we will indicate as PIVKA-II, PIVKA-VII, PIVKA-IX, and PIVKA-X, respectively.

One tends to have a low opinion of kinetic evidence, such as we used to claim the existence of PIVKA. In any case, the postulation of new proteins makes it compulsory to purify and characterize them.

The first independent evidence that something like PIVKA-II actually existed was provided by Josso, who found that under oral anticoagulation in the human there are two populations of molecules reacting with antibodies against prothrombin (3, 4). One of these populations was indistinguishable from normal

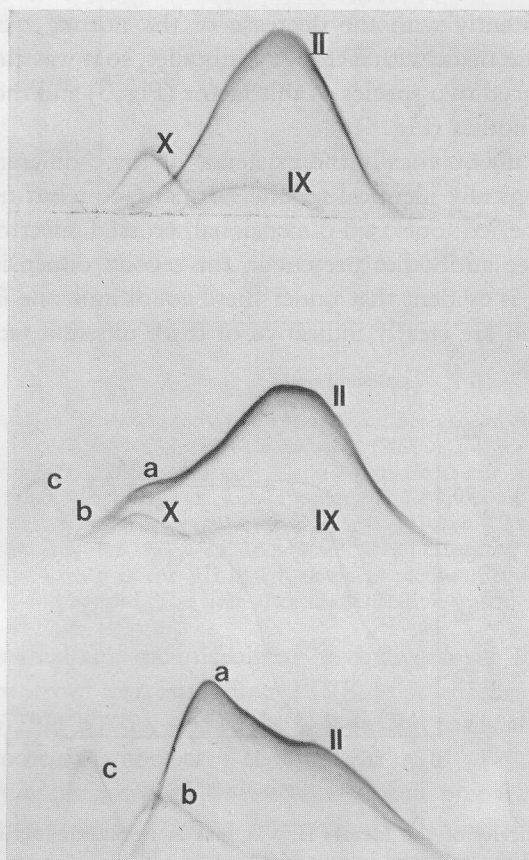


Fig. 5. Two-dimensional crossed electrophoresis of bovine plasmas against a mixture of anti-factor II, anti-factor IX, and anti-factor X, present in the second (vertical) dimension. Top: normal plasma. Middle: plasma after 24 h of anticoagulation. Bottom: plasma after 100 h of anticoagulation.

The figures II, IX, and X indicate the peaks obtained with the normal factors, the letters a, b, and c the corresponding fast-moving proteins.

prothrombin; the other had a faster anodic mobility in the presence of Ca^{++} ions, presumably because they were unable to bind these ions. For prothrombin, in both the human and the cow, this was later confirmed by other workers (5, 6, 7). To demonstrate the existence of other PIVKAs, we followed a comparable experimental procedure. We purified bovine factors II, VII, IX, and X and injected them into rabbits. We failed to obtain an antibody against factor VII, but we did obtain monospecific antibodies against factors II, IX, and X (Fig. 1). With the aid of anti-factor II we were able to confirm the existence of two populations of factor II-like molecules under oral anticoagulation, the abnormal prothrombin

increasing concomitantly with the decrease of the normal factor (Fig. 2). The same observation was made with factor X antibodies, so it was clear that vitamin K deficiency also induced two species of this factor (Fig. 3) and the same was found with factor IX antibodies (Fig. 4).

To demonstrate unequivocally the existence of three different PIVKAs, each of them immunologically identical to one normal factor but non-identical with each other, we carried out two-dimensional crossed electrophoresis with a mixture of the three antibodies present in the second dimension. The result is shown in Fig. 5. It is evident that under these conditions one finds three sets of double peaks, which are clearly indicative of three different factors each having its own PIVKA (8).

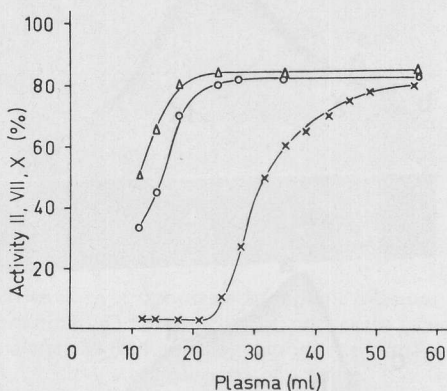


Fig. 6. Assay of the activity of the factors II, VII, and X in plasma leaving an anti-X-immunoadsorbent column. 100 ml normal bovine plasma was added. The first 10 ml coming out of the column were discarded because of dilution with buffer. ○—○, factor II; △—△, factor VII; ×—×, factor X.

The monospecific antibodies can also be used for the purification of both the normal factor and the corresponding PIVKA (9). The antibody is immobilized in a polyacrylamide matrix according to Carrel and Barandun (10) and a column is prepared with this material. We shall illustrate this for factor X only, but completely comparable results are obtained for factors II and IX. When normal plasma is applied to such a column, the factor X activity is retarded (Fig. 6). After the column is washed with buffer containing 3 M KCNS, the factor X activity is eluted. In this way a very pure factor X preparation is obtained from normal plasma. From a deeply anticoagulated cow (factor X activity < 5%), an apparently homogeneous protein is obtained that has no coagulation activity (Fig. 7, Table 2). In crossed immunoelectrophoresis, a mixture of the two purified proteins gives a picture that is completely comparable with that obtained with a

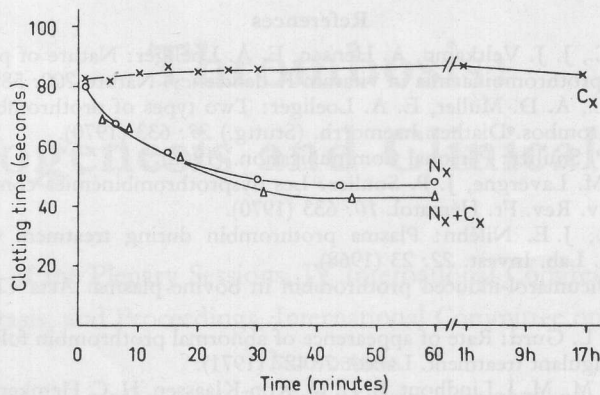


Fig. 7. Generation of factor X_a-activity by Russell's viper venom (RVV) from normal factor X (180 μ g/ml, $\circ - \circ$, curve N_X), coumarin factor X (200 μ g/ml, $\times - \times$, curve C_X), and a mixture of equal amounts of normal factor X and coumarin factor X ($\triangle - \triangle$, curve N_X + C_X). Incubation mixture: 1.8 ml veronal acetate buffer pH 7.35, 0.2 ml CaCl₂ 50 mM, 0.1 ml RVV (50 μ g/ml) and 0.1 ml factor X preparation (N_X or C_X); when 0.2 ml of mixture N_X + C_X was incubated, 1.7 ml of buffer was used. Incubation was performed at 37° C for various hours. Subsampling was done in factor VII and X deficient medium, containing inosithin as a phospholipid. The time after recalcification was measured.

mixture of normal and dicumarolized bovine plasmas. It is possible to separate normal factor from corresponding PIVKAs with the aid of Al(OH)₃. Like PIVKA-II (6), PIVKA-IX and PIVKA-X are less easily absorbable onto Al(OH)₃ than are the normal factors.

Preliminary experiments with the six different preparations obtainable in this way demonstrate one common N-terminal: alanine. Preliminary experiments with antihuman factors II, IX, and X seem to duplicate completely the results obtained in the cow.

In conclusion, it can be said that we have been able to demonstrate and purify three different types of proteins induced by vitamin K antagonists, corresponding to the normal factors II, IX, and X, respectively.

Table 2. Factor X preparations from an immunoabsorbent column.

Starting plasma		Preparation			Electrophoresis
	act. %	act. %	prot. mg/ml	spec. act. U/mg	
Normal (n = 8)	100	57	.79	.95	Homogeneous
Coumarin	25	4	.21	.20	Two peaks
Coumarin	10	5	.40	.12	Two peaks
Coumarin	9	2	.18	.11	Two peaks
Coumarin	8	5	.61	.08	Two peaks
Coumarin	1	1	.45	—	Homogeneous

References

(1) Hemker, H. C., J. J. Veltkamp, A. Hensen, E. A. Loeliger: Nature of prothrombin biosynthesis: preprothrombinaemia in vitamin K deficiency. *Nature* 200: 589 (1963).

(2) Hemker, H. C., A. D. Muller, E. A. Loeliger: Two types of prothrombin in vitamin K deficiency. *Thrombos. Diathes. haemorrh. (Stuttg.)* 23: 633 (1970).

(3) Josso, F., J. P. Soulier: Personal Communication. (1968).

(4) Josso, F., J. M. Lavergne, J. P. Soulier: Les dysprothrombinémies constitutionnelles et acquises. *Nouv. Rev. Fr. Hématol.* 10: 633 (1970).

(5) Ganrot, P. O., J. E. Niléhn: Plasma prothrombin during treatment with dicumarol. *Scand. J. Clin. Lab. Invest.* 22: 23 (1968).

(6) Stenflo, J.: Dicumarol-induced prothrombin in bovine plasma. *Acta Chem. Scand.* 24: 3762 (1970).

(7) Brozovic, M., L. Gurd: Rate of appearance of abnormal prothrombin following initiation of oral anticoagulant treatment. *Lancet* 2: 427 (1971).

(8) Reekers, P. P. M., M. J. Lindhout, B. H. M. Kop-Klaassen, H. C. Hemker: Demonstration of three anomalous plasma proteins induced by a vitamin K antagonist. *Biochim. Biophys. Acta* 317: 559 (1973).

(9) Reekers, P. P. M.: Proteins induced by vitamin K absence in the cow. Thesis, Leiden, 1972.

(10) Carrel, S., S. Barandun: Protein containing polyacrylamide gels, their use as immuno-adsorbents of high capacity. *Immunochemistry* 8: 39 (1971).

regime of normal and dicumarolized bovine plasma. It is possible to separate normal factor from corresponding PIVKs with the aid of Al(OH)_3 . Like PIVK-II (c), PIVK-IX and PIVK-X are less easily absorbable onto Al(OH)_3 than are the normal factors.

Preliminary experiments with the six different preparations obtainable in this way demonstrate one common N-terminal: alanine. Preliminary experiments with antiserum factors II, IX, and X seem to duplicate completely the results obtained in the cow.

In conclusion, it can be said that we have been able to demonstrate and purify three distinct types of proteins induced by vitamin K antagonists, corresponding to the normal factors II, IX, and X, respectively.

Table 2. Factor X preparation from an immunodepleted plasma

Starting plasma	Fractionation			Electrophoresis
	act. %	proc. against spec. act. Unit	act. %	
Normal (n = 8)	100	57	79	95
Common	25	4	31	36
Common	10	2	40	12
Common	9	2	16	11
Common	8	2	51	45
Common	1	1	45	—